

Cloning structural genes for *Treponema pallidum* immunogens and characterisation of recombinant treponemal surface protein, P2* (P2 star)

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SUMMARY A genomic library consisting of partially digested 10 to 20 kilobase pair fragments of *Treponema pallidum* deoxyribonucleic acid (DNA) was constructed using bacteriophage lambda EMBL-3 as the vector. Positive clones expressing *T pallidum* antigens were detected with sera from experimentally infected rabbits. Treponemal proteins ranging in molecular weight from 37 000 daltons to 120 000 daltons were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblotting of phage lysate proteins. One recombinant phage was examined further and contained an insert encoding a prominent treponemal 37 000 dalton protein. The recombinant protein was not recognised by antiserum directed against a fibronectin binding treponemal adhesin that contained the same electrophoretic mobility. Neither did antibody to the recombinant 37 000 dalton protein react with any treponemal proteins purified by fibronectin affinity chromatography. The recombinant protein in *Escherichia coli* lysates was labelled P2* (P2 star) to differentiate it from the comigrating adhesin protein called P2. Native P2* protein was present on *T pallidum* surfaces as shown by radioimmunoprecipitation assays with extrinsically labelled organisms. A cross reactive molecule like P2* was not synthesised by the avirulent spirochaete, *T phagedenis* biotype Reiter, which indicated that P2* is a protein specific to virulent *T pallidum* organisms. Finally, only sera of patients with primary syphilis possessed appreciable concentrations of antibody to recombinant P2* protein.

Infection with *Treponema pallidum* elicits the production of "protective" antibodies that reduce, delay, or abrogate the development of lesions in experimental animals.¹⁻⁵ The function of these immunoglobulins in protection is uncertain, as organisms persist in the presence of high titred antibodies to *T pallidum*.³ The role of cell mediated immune effectors in response to *T pallidum* infection is also poorly understood.⁶

An active area of research in recent years has been the evaluation of host humoral responses to specific treponemal components during syphilis.⁷⁻¹² Radioimmunoprecipitation electrophoresis and immunoblot assays, for example, have shown numerous immunogenic treponemal proteins. The biological

function and location of these immunogens, however, have not been assessed, partly because this pathogen is not satisfactorily cultivable *in vitro*.¹³ Recombinant deoxyribonucleic acid (DNA) technology¹⁴⁻¹⁸ is therefore necessary to obtain sufficient quantities of parasite antigen.¹⁹ This may permit a detailed analysis of the role, if any, of precise treponemal proteins in *T pallidum* virulence and in the pathogenesis of syphilis. In this study, we describe the cloning and expression of several genes for treponemal proteins. We show the highly immunogenic nature and surface disposition of a recombinant 37 000 dalton protein, and discuss our results in terms of the biology of the syphilis spirochaete.

Materials and methods

BACTERIA

The Nichols strain of *T pallidum* was inoculated intratesticularly into New Zealand white rabbits as described previously.²⁰ Treponemes were harvested

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from minced testicular tissue excised at peak orchitis (7–11 days after inoculation) by shaking in 15 ml of a salts-glucose medium under reducing conditions for 20 minutes at 34°C.^{7 21 22} The treponemal suspension containing 1×10^8 to 4×10^8 organisms/ml was centrifuged twice at room temperature at $500 \times g$ for 15 minutes followed by centrifugation on a cushion of 0.8% Methacel (Dow Corning, Midland, Mississippi, USA) and 50% Hypaque (Winthrop Labs, New York) at $650 \times g$ for 20 minutes. The supernate containing highly motile treponemes was centrifuged at $17000 \times g$ to pellet the organisms. The treponemal pellets were stored at -70°C until needed for DNA extraction or for preparing total proteins.²³

Escherichia coli strains LE 392²⁴ (F^- , *hsdR514*, *supE44*, *supF58*, *lacY*, *galK2*, *galT22*, *metB*, *trpR55*, λ mbda⁻) and NM539²⁵ (*supF*, *hsdR* [P2 *cox*]) (Promega biotec) were grown in Luria broth.²⁴

SERA

Serum obtained from rabbits inoculated intratesticularly with live treponemes^{2 7 22} was extensively adsorbed with *E coli* LE 392 before being used for clone bank screening. Serum samples from patients with syphilis were gifts from Sandra Larsen of the Center for Disease Control, Atlanta, Georgia, and have been characterised by us.²⁶ Serum samples from uninfected rabbits and from people without a history of sexually transmitted diseases were used as controls.

DNA EXTRACTION FROM *T PALLIDUM*

About 1×10^{11} washed pelleted spirochaetes were resuspended in 5 ml of 50 mmol/l TRIS and hydrochloric acid (HCl) buffer, pH 8.0, containing 50 mmol/l ethylenediaminetetra-acetic acid (EDTA).²⁷ A volume of 5 mg lysozyme in 0.5 ml of 250 mmol/l TRIS-HCl, pH 8.0, was then added, and the reaction kept on ice for 45 minutes. Next, 1 ml of disrupting buffer (0.5% sodium dodecyl sulphate (SDS), 50 mmol/l TRIS-HCl, 400 mmol/l EDTA, and 1 mg/l proteinase K), pH 7.5, was added, and the mixture was incubated for one hour at 50°C with occasional shaking. This material was then extracted three times with TRIS buffered phenol.²⁴ The aqueous layer was precipitated with 15 ml cold ethanol in 300 mmol/l sodium acetate. The precipitate was resuspended in 5 ml TRIS-EDTA buffer (50 mmol/l TRIS-HCl and 1 mmol/l EDTA), pH 8.0, containing 200 µg DNase free RNase/ml (Boehringer Mannheim). After overnight incubation at 4°C, the material was extracted twice with 5 ml chloroform, and the DNA was precipitated with ethanol and sodium acetate.²⁴ Finally, the DNA was resuspended in TRIS-EDTA buffer, pH 8.0, at a concentration of 1 mg/ml.

CONSTRUCTION OF *T PALLIDUM* GENOMIC LIBRARY

A bacteriophage lambda library of *T pallidum* DNA was prepared as described in fig 1. Briefly, *T pallidum* DNA was partially digested with BamHI restriction endonuclease (Boehringer Mannheim) and fractionated by centrifugation on a linear 10% to 40% sucrose gradient.²⁴ DNA fragments containing 10 to 20 kilobase pairs were pooled and dialysed against TRIS-EDTA buffer, pH 8.0, followed by phenol extraction²⁴ and ethanol precipitation.²⁴

EMBL-3 vector DNA digested with BamHI and EcoRI endonuclease was ligated to the sized *T pallidum* fragments and packaged to produce viable phages using a lambda in vitro packaging system (Promega Biotec).

IMMUNOLOGICAL SCREENING OF CLONE BANK

Recombinant phage plaque material was immobilised on nitrocellulose discs. These discs were placed in TRIS buffered saline (500 mmol/l sodium chloride, and 20 mmol/l TRIS-HCl) with 1% bovine serum albumin, pH 7.4, for two hours at room temperature and then incubated with adsorbed syphilitic rabbit serum at 1:50 dilution in TRIS buffered saline and 5% non-fat dried milk.^{14 28} The nitrocellulose was washed three times for 15 minutes with TRIS buffered saline, and horseradish peroxidase conjugated goat anti-rabbit IgG (BioRad) diluted 1:2000 in TRIS buffered saline and 5% milk was then added. The blots were washed three additional times before substrate was added. Positive clones were spotted in duplicate on *E coli*, transferred to nitrocellulose, and tested again for antibody reactivity to insure the isolation of clones producing positive signals.

RADIOIMMUNOPRECIPITATION ASSAY, SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE), AND IMMUNOBLOTTING

Detergent solubilised treponemal proteins were radioimmunoprecipitated under conditions detailed previously.⁷ Protein preparations subjected to SDS-PAGE were immunoblotted as described previously.^{7 14 21 22 29} Proteins from recombinant *E coli* were prepared for SDS-PAGE by scraping the soft agarose overlays from 1 cm diameter phage plaques and boiling them in SDS-PAGE dissolving buffer.

ELUTION OF ANTIBODY TO *T PALLIDUM* FROM PHAGE LYSATES

Nitrocellulose discs containing proteins from plate lysates of individual recombinant clones were incubated overnight at 4°C with adsorbed syphilitic rabbit serum and then washed three separate times with TRIS buffered saline. Bound antibodies were eluted

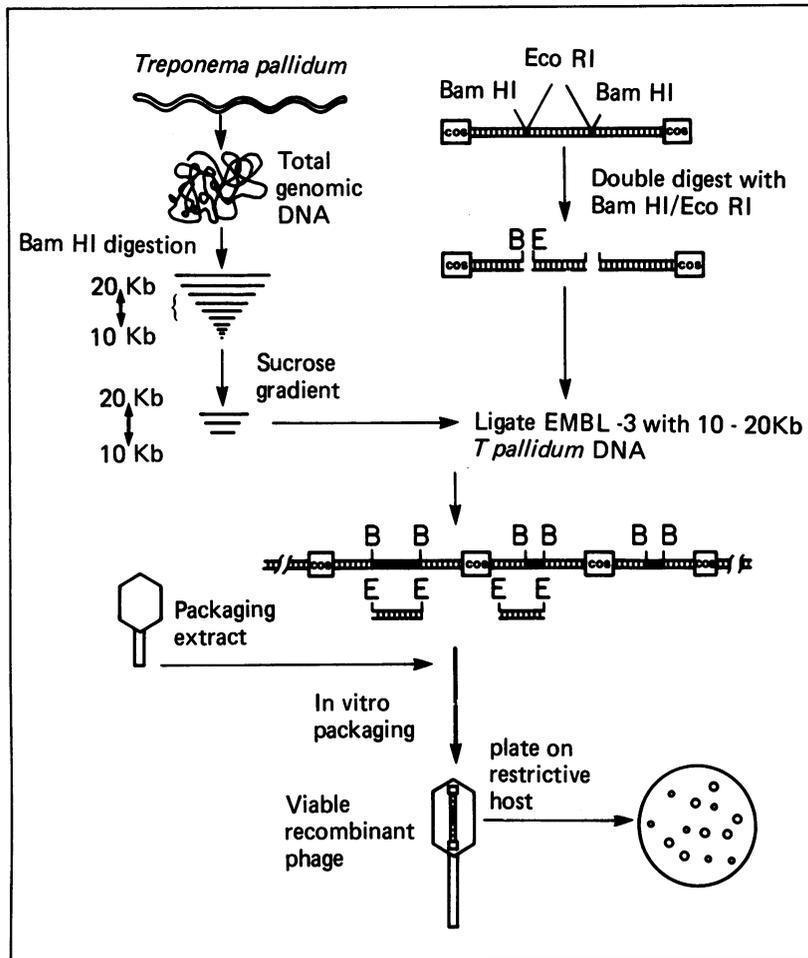


Fig 1 Schematic representation of cloning strategy used to generate *Treponema pallidum* library.

with 5 ml of a solution of 100 mmol/l glycine, 20 mmol/l magnesium acetate, and 50 mmol/l potassium chloride, pH 2.6, for two hours at room temperature and dialysed for 12 hours against two changes of TRIS buffered saline before being stored at -20°C .¹⁴

DOT BLOT ANALYSIS OF B5 RECOMBINANT PHAGE EXPRESSING P2*

Phages were spotted on to *E coli*, transferred to nitrocellulose, and tested for reactivity to serum from patients with syphilis.²⁶ Individual nitrocellulose immobilised plaques were cut into 2 cm squares and placed in 24 well culture plates. A volume of 1 ml human syphilitic serum diluted 1:50 in TRIS buffered saline and 5% milk was added to the squares followed

by 100 μl *E coli* disrupted in a French pressure cell. Incubation was carried out at 4°C overnight, followed by three washes with TRIS buffered saline. Horseradish peroxidase conjugated goat anti-human IgG (Cappel, Cochranville, Pennsylvania) diluted 1:500 in TRIS buffered saline and 5% milk was added for three hours at 4°C . The dot blots were washed again and developed as described above. Reactivity was compared with that of control squares incubated with second antibody, alone or with normal human serum.

PURIFICATION OF TREPONEMAL PROTEINS BINDING TO FIBRONECTIN

Treponemal proteins P1, P2, and P3 were purified by fibronectin-Sepharose affinity chromatography as described previously.²³ Briefly, 2×10^{10} freshly har-

vested spirochaetes were resuspended in 100 μ l of 10% Zwittergent 3-12 (Z (3-12); Calbiochem-Behring) detergent.²³ After being homogenised and clarified, the extract was diluted in phosphate buffered saline (PBS) to give a final concentration of 0.05% Z(3-12). This soluble preparation was then incubated with 500 μ l of fibronectin-Sepharose²³ for six hours at room temperature with gentle rocking. The beads were then washed with 100 volumes of PBS and 0.05% Z(3-12). Treponemal material adherent to fibronectin (P1, P2, and P3) was released by boiling in 1 ml of electrophoresis solubilising buffer⁷ for three minutes. The Sepharose was pelleted by centrifugation, and the resulting supernate subjected to SDS-PAGE for immunological analysis.

Results

ESTABLISHING *T PALLIDUM* GENOMIC LIBRARY Figure 1 shows the scheme employed for obtaining a genomic library of *T pallidum* DNA. We chose EMBL-3 bacteriophage as a cloning vehicle for *T pallidum* DNA because of its high cloning efficiency, its large insert size (10-20 kilobase pairs), and its ability to minimise non-recombinant background phages. This system generated a library of over 100 000 recombinant phages for further analysis.

Figure 2 shows an immunoblot of the recombinant treponemal proteins from representative phage clones producing signals. Some clones produced several distinct treponemal polypeptides, as evidenced by mul-

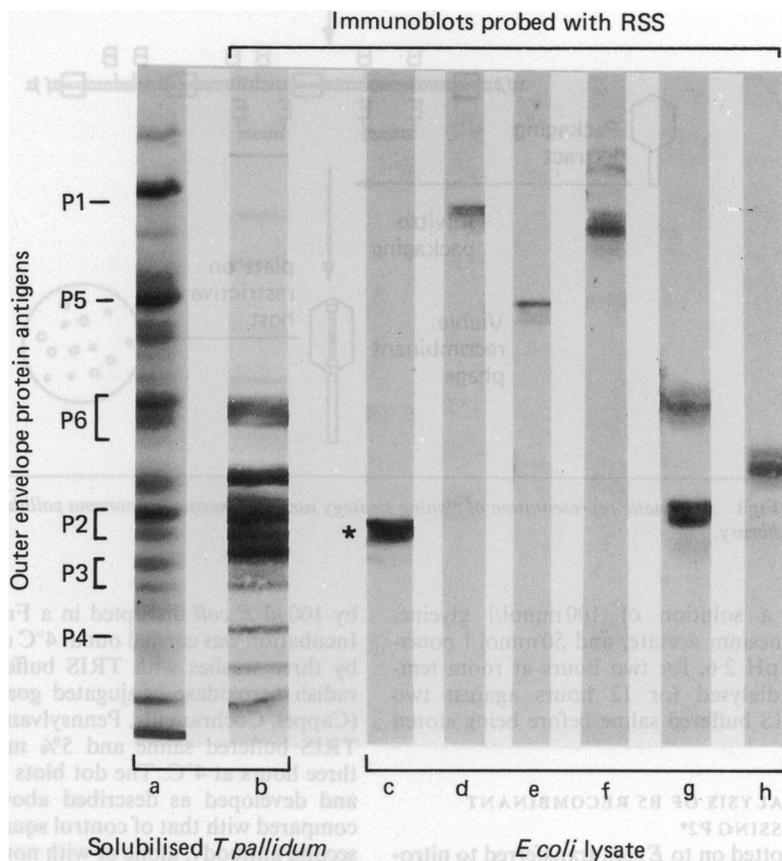


Fig 2 Immunoblot identification of *Treponema pallidum* proteins present in recombinant phage plaques. Profile of solubilised treponemal proteins stained with Coomassie brilliant blue after SDS-PAGE (lane a). Same proteins transferred to nitrocellulose for detection by syphilitic rabbit serum (lane b). Recombinant protein bands from lysates of individual phage clones reactive with syphilitic rabbit serum adsorbed with *Escherichia coli* (lanes c-h). * = protein of clone B5 called P2* (P2 star).

tiple bands from the same lysate preparation using adsorbed syphilitic rabbit serum (lanes d, f, and g), whereas other lysates yielded a single protein band. Adsorbed syphilitic rabbit serum did not recognise any rabbit testicular tissue proteins or control phage lysates. It was important that syphilitic rabbit serum antibodies eluted from individual nitrocellulose blots of these six phage lysates (fig 2, lanes c to h) detected *T pallidum* proteins with the same electrophoretic mobilities. In control experiments, syphilitic rabbit serum antibodies eluted from non-recombinant phage blots detected no treponemal proteins. Normal rabbit serum did not react with recombinant phage lysates or immunoblots of total treponemal proteins. These data, therefore, show the cloning of structural genes for these various *T pallidum* proteins using these experimental conditions.

ANALYSIS OF RECOMBINANT 37000 DALTON *T PALLIDUM* PROTEIN DESIGNATED P2*

We then characterised further a 37000 dalton recombinant protein of phage clone B5 (fig 2, lane c) because it possessed a relative molecular weight identical to that of the treponemal proteins that bound to fibronectin, which had been designated P2 in earlier studies.²¹⁻²³ Antibodies were eluted from nitrocellulose blots of phage lysates containing the 37000 dalton recombinant protein. The eluted antibodies, however, failed to recognise by immunoblot any of the purified treponemal adhesins (data not shown).²³ The recombinant protein also failed to bind to fibronectin-Sepharose,²³ and antibodies to the treponemal fibronectin binding adhesins did not react with the 37000 dalton recombinant protein. These data indicated that the recombinant 37000 dalton protein designated P2* can be differentiated from the comigrating adhesin molecule P2.²³

Native *T pallidum* was then compared with the recombinant P2* proteins. As can be seen in fig 3, syphilitic rabbit serum antibody eluted from nitrocellulose blots of phage lysates with P2* detected a single band in the total treponemal protein preparation (lane c). It also identified a protein band of the same size as recombinant P2* (lane b). Syphilitic rabbit serum adsorbed on nitrocellulose blots containing P2* phage lysate also resulted in decreased reactivity to the 37000 dalton migrating region of the immunoblot (lane e). Syphilitic rabbit serum adsorbed with control phage lysates was unchanged (lane d), and, as expected, normal rabbit serum did not react with the recombinant 37000 dalton protein or treponemal proteins.⁷ These data show the immunogenic nature of P2* and confirm the cloning of the structural gene for this protein antigen.

DEMONSTRATION OF THE SURFACE LOCATION OF NATIVE P2*

Figure 4 shows the radioimmunoprecipitation of radioiodinated P2* from a detergent extract of iodinated²¹ treponemes (lane d) using antibody eluted from phage blots containing recombinant 37000 dalton protein. Furthermore, syphilitic rabbit serum adsorbed against blots of recombinant P2* showed diminished band intensity for iodinated P2* when compared with other precipitated surface proteins (lane c) or when unadsorbed syphilitic rabbit serum was used (lane b). It was important that eluted antibody adsorbed against live *T pallidum* organisms resulted in no detectable iodinated P2* (data not shown), confirming the surface disposition of this treponemal protein. As expected, normal rabbit serum did not immunoprecipitate iodinated treponemal proteins.

REACTIVITY OF HUMAN SYPHILITIC SERA WITH RECOMBINANT P2*

We then tested by immunoblot analysis various serum samples from patients with primary, secondary, and latent syphilis and control samples from uninfected people for their reactivity to recombinant 37000 dalton protein. Using 1:10 dilutions of the various sera, those from five out of six patients with primary syphilis were highly reactive and one was weakly reactive with recombinant P2*, whereas only one of five with secondary syphilis and none of six with latent syphilis possessed antibody to detect this treponemal surface component.

Discussion

In this report we show the generation of a gene library of *T pallidum* DNA in bacteriophage lambda in which recombinant treponemal proteins were recognised by antibodies from experimentally infected animals. The observation that antibodies affinity purified from nitrocellulose blots of recombinant proteins recognised *T pallidum* proteins of the same electrophoretic mobility (fig 3) suggested that the intact structural genes for these respective treponemal products had been cloned.

A phage clone encoding the 37000 dalton polypeptide designated P2* was chosen for further study because it possessed a molecular mass similar to that of a treponemal adhesin previously implicated as a *T pallidum* fibronectin binding protein.²³ Antibodies specific for the recombinant 37000 dalton protein, however, did not recognise *T pallidum* adhesins isolated by fibronectin affinity chromatography. Thus the recombinant 37000 dalton protein represents a distinct immunogen with the same electrophoretic mobility as the adhesin, which suggests that antibody

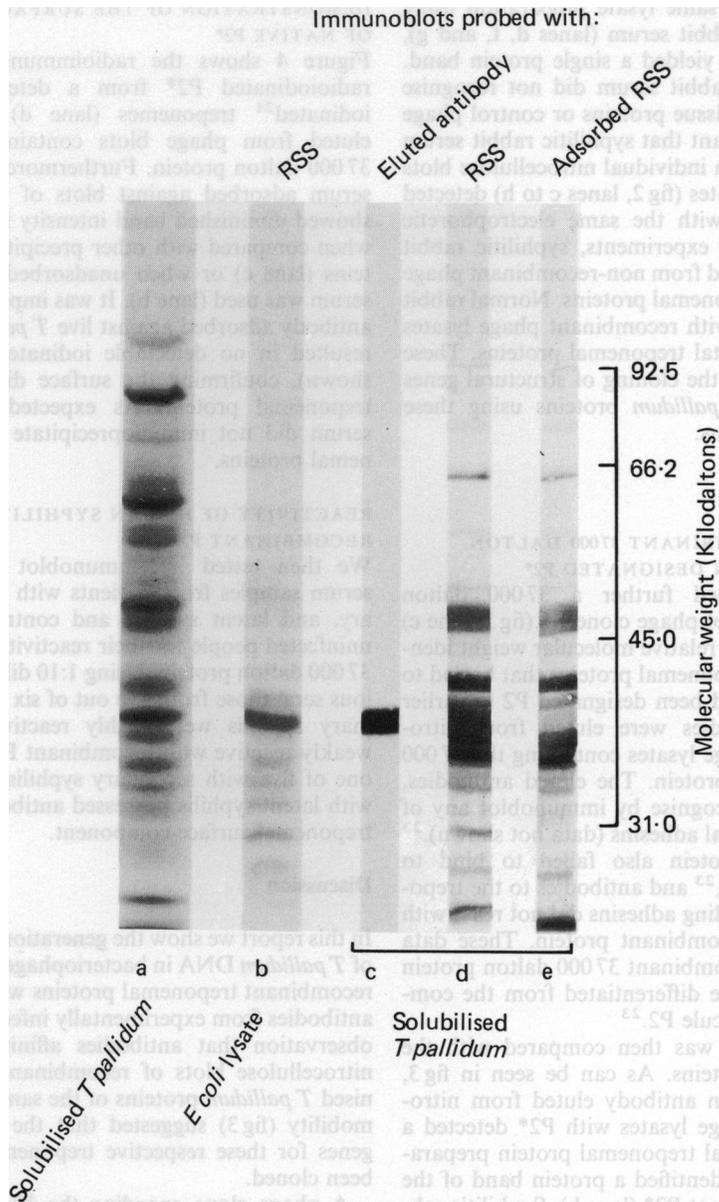


Fig 3 Immunoblot analysis of recombinant 37 000 dalton *Treponema pallidum* protein P2*. Stained protein pattern of solubilised *T pallidum* proteins after SDS-PAGE (lane a). Recombinant P2* phage product in *Escherichia coli* lysates detected by rabbit syphilitic serum (lane b). Treponemal protein identified by antibodies eluted from recombinant phage plaques containing P2* (lane c). *T pallidum* protein antigens detected by syphilitic rabbit serum (lane d) and by syphilitic rabbit serum adsorbed against nitrocellulose blots containing recombinant P2* protein (lane e).

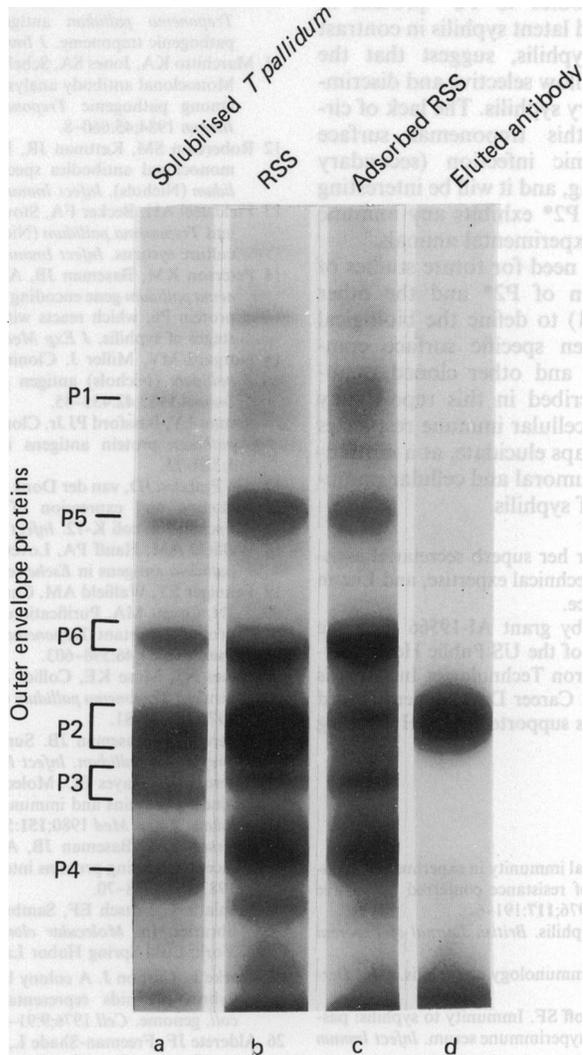


Fig 4 Surface location of P2* shown by radioimmuno-precipitation assay. Surface radioiodinated *T pallidum* were solubilised with detergent and incubated with rabbit syphilitic serum (lane b), syphilitic rabbit serum adsorbed against nitrocellulose blots of phage lysates containing P2* (lane c), and antibody to syphilitic rabbit serum eluted from blots of recombinant P2* (lane d). Autoradiogram of detergent solubilised *T pallidum* proteins (lane a).

reactive proteins of this molecular weight are not solely the adhesins as suggested previously,^{7,30} thus differentiating the rabbit and human antibody produced during infection with P2* from that produced by other comigrating immunogens.

Consistently with recent studies,^{7,8,10} however, these data show that the 37000 dalton recombinant protein called P2* does represent a prominent immu-

nogenic surface component (figs 3 and 4). The fact that a similar protein was found to be synthesised by *T pertenuis* but not by the avirulent spirochaete, *T phagedenis* biotype Reiter (data not shown), supports the idea that P2* and other molecules, such as the adhesins,³⁰ are virulence markers for these pathogens.

The initial data, which show by immunoblot the

relative absence of antibodies to P2* protein in patients with secondary and latent syphilis in contrast to those with primary syphilis, suggest that the recombinant protein may allow selective and discriminatory diagnosis of primary syphilis. The lack of circulating antibodies to this treponemal surface component during systemic infection (secondary syphilis) is in itself intriguing, and it will be interesting to assess whether purified P2* exhibits any immune modulating properties in experimental animals.⁶

These data illustrate the need for future studies of the structure and function of P2* and the other recombinant proteins (fig 1) to define the biological properties of the pathogen specific surface components. Isolation of P2* and other cloned treponemal gene products described in this report may facilitate an evaluation of cellular immune responses to select antigens and perhaps elucidate, at a molecular level, the influence of humoral and cellular immunity on the pathogenesis of syphilis.

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